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Plant regeneration from cotyledons of *Prunus persica*, *Prunus domestica*, and *Prunus cerasus*

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Abstract. Shoots were regenerated from the proximal region of immature cotyledons (with the embryonic axis removed) of *Prunus persica* (peach) and from the same area in mature cotyledons of *P. domestica* (plum) and *P. cerasus* (sour cherry) on MS medium containing (in $mg\,l^{-1}$) thiamine-HCl, 0.4; nicotinic acid, 0.5; pyridoxine-HCl, 0.5; sucrose, 25 000; and 0.7% agar. The medium was supplemented with 0.0–2.5 μM indole-butyric acid and 5–12.5 μM thidiazuron. Cultures were incubated at 24 °C under 16 h photoperiod. Shoots regenerated adventitiously over a broad range of thidiazuron concentrations and 2.5 μM indole-butyric acid in 35 days. The presence of the embryonic axis inhibited the development of shoots. Regenerated shoots of peach and plum were rooted on half-strength MS inorganic semi-solid medium with 2.5–5.0 μM indole-butyric acid. Rooted plants were acclimatized and transferred to the greenhouse.

Abbreviations: BAP – 6-benzylaminopurine; IBA – indole-butyric acid; TDZ – N-phenyl-N-1,2,3-thidiazol-5-ylurea; IAA – indole-acetic acid; NAA – α-naphthalene-acetic acid

Introduction

Plant tissue culture methodology is well established for ornamental as well as herbaceous plants [8]. However, to date, investigators have made relatively slow progress on plant regeneration protocols for tree species. The genus *Prunus* includes ornamental, fruit-bearing, and hardwood tree species. In vitro plant regeneration has been variably successful with the fruit-bearing species. Colt, an interspecific (*P. avium* × *P. pseudocerasus*) cherry rootstock, has been successfully regenerated from protoplasts [10]. There have been reports of sporadic shoot regeneration from callus cultures derived

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from *P. amygdalus* (almond) cotyledons and leaves [6]. Other sources of calli from which plants have been developed include anthers of *P. cerasus* [13] and roots of *P. dawyckensis* and *P. canescens* as well as those of *P. incisa* × *serrula* [1, 2]. Culture of immature embryos (or cotyledons) of *P. avium* (sweet cherry), *P. armeniaca* (apricot) [5] and *P. persica* (peach) on various media, have produced adventitious shoots in vitro [4]. However, the use of immature embryos with regeneration potential is limited to short periods of time each year [4], and successful protocols for shoot regeneration from vegetative sources are yet to be developed.

The cytokinins 6-benzylaminopurine (BAP), kinetin, zeatin, or isopentenyladenine have been used to stimulate shoot regeneration in *Prunus*, but the numbers of shoots produced per explant have been relatively low [5, 6]. Thidiazuron (N-phenyl-N-1,2,3-thidiazol-5-ylurea, (TDZ) is a compound with cytokinin-like activity which has been found to induce shoot development in a number of plant tissue culture systems [3, 7, 14]. Its use has not, to our knowledge, been reported for *Prunus* tissue culture.

In this study, we report TDZ-induced in vitro plant regeneration from mature, stored cotyledons of *P. cerasus* (sour cherry) and *P. domestica* (European plum) and regeneration from immature, stored cotyledons of *P persica* (peach).

Materials and methods

Plant materials

Peach fruits from the cultivars 'Boone County', 'Bailey', 'Belle', 'Loring', unnamed clingstone, 'Suncrest', the peach × almond hybrid 'Hann' and those of European plum cultivar 'Stanley' and plum breeding selection B70173 were collected from the USDA-ARS Appalachian Fruit Research Station orchard. The peach and 'Hann' fruits were collected at 70, 100–110, and 130 days after full bloom, and stored at 4 °C and used over a 90-day period. Sour cherry seeds ('Montmorency', open-pollinated) were obtained from Dr. Susan Brown, New York State Agricultural Experiment Station, Geneva, NY. The seeds of plum and sour cherry were mature and stored at 4 °C and used over a 90-day period.

Media

Murashige & Skoog (MS) inorganic salts [9] were supplemented with (in $mg\,l^{-1}$): I-inositol, 100; thiamine-HCl, 0.4; nicotinic acid, 0.5; pyridoxine-

HCl, 0.5; sucrose, 25 000; and 0.7% tissue culture (TC) agar (KC Biological, Denver, PA, USA). This basal medium was supplemented with α-naphthalene-acetic acid (NAA), indole-butyric acid (IBA), or indole-acetic acid (IAA), and cytokinin (BAP, or TDZ) as specified below. TDZ was obtained from NOR-AM Chemical Co., Wilmington, DE, USA. A stock solution was made in 10 ml 1N sodium hydroxide (NaOH), made up to volume with de-ionized water and stored at 4 °C until used. BAP, IBA, and NAA were added to the media before autoclaving, and IAA and TDZ were filter-sterilized and added to the media after autoclaving. Media were adjusted to a pH of 6.05–6.10 with 1N NaOH or 1N HCl and autoclaved at 1.4 kg cm^{-2} for 20 min. Media were dispensed into 100 × 20 mm Petri dishes (55 ml each).

Explant preparation

Peach fruits were split open and the seeds were removed, washed under running tap water for about 5 min, and disinfected with 0.5% sodium hypochlorite (10% commercial bleach with a few drops of 1% Triton × 100) for 12–15 min. All further manipulations were done under sterile conditions. The seed coat was removed, the cotyledons split apart, and the embryonic axis removed. Each cotyledon was explanted on treatment medium (Fig. 1, left). The endocarps of sour cherry and plum were cracked open and the seeds were disinfected as described above. The disinfected seeds were soaked in sterile de-ionized water overnight, and the cotyledons cultured as described for peach.

Culture conditions

The explants were cultured under 45–50 μE m^{-2} s^{-1} of mixed warm-white fluorescent (General Electric) and Vita-lite full-spectrum fluorescent (Duro Test Corp.) lamps at 24 °C with 16 h photoperiod. Each experiment was repeated 2 times with 20 cotyledons (10 seeds) in each treatment. While explant surface orientation ab- or abaxial to the medium did not appear to influence morphogenesis, it was essential that the cotyledon surface was always in complete contact with the medium. Explants with slow growing shoot initials were subcultured onto the same medium. Regenerated shoots were excised and rooted on half-strength MS inorganic medium containing vitamins, 2% sucrose, 2.5–5.0 μM IBA, and 0.7% TC agar at 24 °C and 16 h photoperiod prior to acclimatization and planting in the greenhouse.

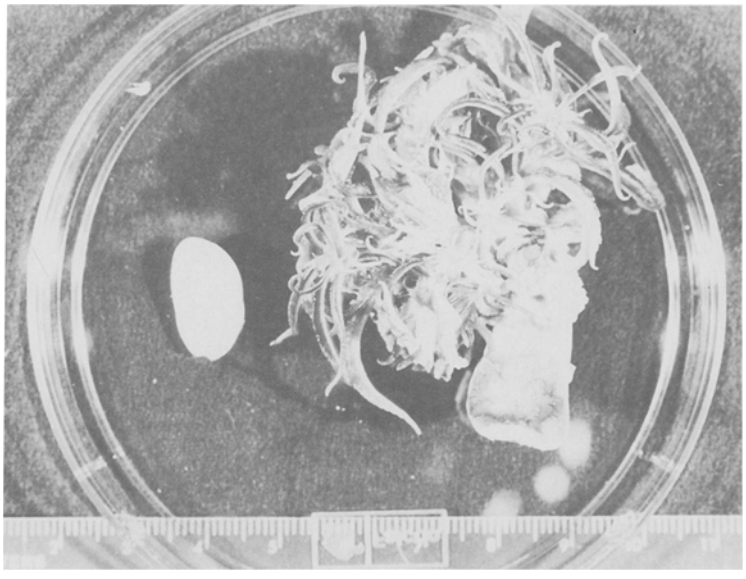

Fig. 1. (*Left*) Initial explant (cotyledon) used for shoot regeneration. (*Right*) Shoot regeneration from peach cotyledon, 35 days after culture initiation on MS-IBA-TDZ medium.

Results

Peach/growth regulator effects

In preliminary tests, cotyledons of 'Bailey', 'Boone County', 'Suncrest', and 'Hann' (70 days after bloom), developed shoots at the proximal end on media containing 2.5 μM IBA and 5.0 μM TDZ within 4–6 weeks (Fig. 1, right). If the embryonic axis was left in place or the proximal region of the cotyledon was removed, no shoots developed. In addition, a cut surface in the distal region of the cotyledon produced no shoots during the culture period. Cotyledons grown on media containing 2.5 μM IBA and 5.0 μM BAP produced roots, with an occasional isolated shoot. Cotyledons did not produce shoots in the presence of NAA or IAA combined with either BAP or TDZ.

Effect of IBA and TDZ concentrations on shoot regeneration from 'Suncrest' 70-day post-bloom peach cotyledons

Twenty cotyledons with their embryonic axes removed, were cultured on MS media supplemented with either 0.5 or 2.5 μM IBA and 5.0–12.5 μM

Table 1. Shoot formation on immature cotyledons isolated from peach cultivar Suncrest fruits, collected 70 days after full bloom, kept at 4°C for 28 days, and cultured on MS media supplemented with different concentrations of IBA and TDZ for 35 days.

Growth regulator (μM)		Percent cotyledons with shoots[1]	Mean number of shoots/ cotyledon[2] $\pm$ SE[3] of mean
IBA	TDZ		
0.5	5.0	50	4.75 $\pm$ 1.7
0.5	7.5	63	7.4 $\pm$ 3.1
0.5	10.0	60	2.7 $\pm$ 0.5
0.5	12.5	0	0.0
2.5	5.0	40	5.0 $\pm$ 1.7
2.5	7.5	59	13.2 $\pm$ 1.9
2.5	10.0	38	6.7 $\pm$ 7.1
2.5	12.5	67	15.5 $\pm$ 7.1
2.5	0.0	0	0.0*
0.0	7.5	20	2.3 $\pm$ 0.7

[1] Based on 15–20 cotyledons
[2] From cotyledons that produced shoots
[3] Standard error
* Occasionally roots developed

TDZ. The explants turned green and enlarged during the first 7 days. These events were followed by the thickening of the proximal region which eventually turned into a ridge-like structure from which shoots developed in 14–56 days. Occasionally, a small amount of callus developed in some of the explants. Shoots were produced over a broad range of IBA and TDZ concentrations (Table 1). IBA appeared to be necessary for shoot development. As shown in Table 2, other peach cultivars also produced shoots at 2.5 μM IBA and 5–10.0 μM TDZ. In all the cultivars tested, shoot regeneration occurred in most cases from one of the two cotyledons present in the seed, but occasionally, both cotyledons (from one seed) produced shoots. Occasionally, in the presence of IBA alone, roots developed.

Effect of cotyledon age on shoot development in peach

Twenty cotyledons of 'Bailey', at two stages of development (70 and 100–110 days after bloom), were cultured on MS media with 2.5 μM IBA and 7.5 μM TDZ. Immature cotyledons (70 days after bloom) showed 50–60% regeneration and produced an average of 7 shoots per cotyledon, while the older cotyledons (100–110 days after bloom) gave 2–5% regeneration and developed an average of about one shoot per cotyledon (data not shown). Mature cotyledons (130 days after bloom) also regenerated poorly.

Table 2. Shoot formation on immature cotyledons isolated from peach fruits, collected 70 days after full bloom, kept at 4°C for 28 days, and cultured on MS media supplemented with 2.5 μM IBA and different TDZ concentrations for 35 days.

Peach cultivar	Growth regulator IBA/TDZ (μM)	Percent cotyledons with shoots[1]	Mean number of shoots/ cotyledon[2] $\pm$ SE[3] of mean
'Loring'	2.5/5.0	42	2.2 $\pm$ 0.5
	2.5/7.5	67	5.8 $\pm$ 1.3
	2.5/10.0	33	5.3 $\pm$ 1.3
'Belle'	2.5/5.0	50	4.8 $\pm$ 1.3
	2.5/7.5	50	9.4 $\pm$ 2.7
Unnamed clingstone	2.5/5.0	60	5.3 $\pm$ 0.8
	2.5/7.5	70	17.9 $\pm$ 2.1
'Boone County'	2.5/5.0	50	2.2 $\pm$ 0.4
	2.5/7.5	60	4.8 $\pm$ 1.3
	2.5/10.0	30	4.0 $\pm$ 1.4

[1] Based on 10–20 cotyledons
[2] From cotyledons that produced shoots
[3] Standard error

Regeneration of shoots from mature P. domestica (European plum), breeding selection B70173

The results with peach cotyledons had indicated that shoots could be regenerated specifically from the proximal region of the explants. It was decided to investigate whether the same was true for plum cotyledons.

Twenty mature cotyledons of *P. domestica* with their embryonic axes removed, were cultured on MS medium containing 2.5 μM IBA and 0.0–12.5 μM TDZ. The explants turned green, enlarged, and started to thicken at the proximal end with visible growth of light-green leaf-like strucutres in 7 days. By 28 days, shoots had developed at the proximal region of the explant. A few of the shoots (3–4) became dominant 4–6 weeks after culture initiation, while the rest were suppressed (Fig. 2). Shoots regenerated from cotyledons at all concentrations of TDZ tested (Table 3). IBA appeared to be necessary for the TDZ stimulation of shoot development. Occasionally, in the presence of IBA alone, roots developed. When cotyledons were left attached to the embryonic axis, shoot development from the cotyledons was completely inhibited (data not presented). The cultivar Stanley gave similar results.

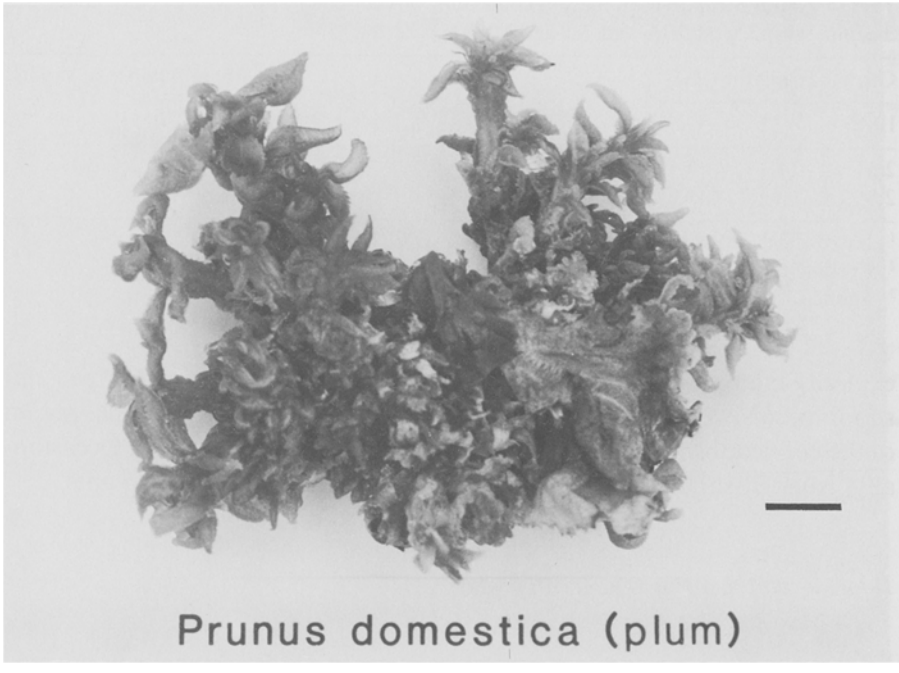

Fig. 2. Shoot regeneration from plum cotyledon, 35 days after culture initiation. Bar = 2 cm

Table 3. Shoot formation on mature cotyledons of *P. domestica* (breeding selection B70173) cultured on MS medium with 2.5 μM IBA and different concentrations of TDZ for 35 days.

Growth regulator (μM)		Percent cotyledons with shoots[1]	Mean number of shoots/ cotyledon[2] $\pm$ SE[3] of mean
IBA	TDZ		
2.5	0.0	0	0.0*
2.5	5.0	76	9.5 $\pm$ 1.3
2.5	7.5	82	13.2 $\pm$ 2.3
2.5	10.0	87	12.6 $\pm$ 1.5
2.5	12.5	85	18.7 $\pm$ 3.4
0.0	7.5	26	4.0 $\pm$ 1.0

[1] Based on 15–20 cotyledons in 2 replications of the same experiment
[2] From cotyledons that produced shoots
[3] Standard error * Occasionally roots developed

Regeneration of shoots from mature P. cerasus (sour cherry) cotyledons

Mature cotyledons of sour cherry with their embryonic axes removed, were cultured on MS medium containing 2.5 μM IBA and 7.5–10.0 μM TDZ. The

Table 4. Shoot formation on mature cotyledons of sour cherry (*P. cerasus*) cultured on MS medium with 2.5 μM IBA and 7.5 or 10.0 μM TDZ for 35 days.

Growth regulator (μM)		Percent cotyledons with shoots[1]	Mean number of shoots/ cotyledon[2] ± SE[3] of mean
IBA	TDZ		
2.5	7.5	58	9.4 ± 1.4
2.5	10.0	64	7.4 ± 1.6

[1] Based on 15–20 cotyledons
[2] From cotyledons that produced shoots
[3] Standard error

explants enlarged within 7 days. By 14 days, they had turned pale green and shoot initials were visible. Shoots were produced from the proximal region of the cotyledons in the presence of both IBA and TDZ (Table 4). Occasionally, isolated shoots developed on the abaxial surface of the explants.

Root development in regenerated plants

Fifteen shoots, each of peach and plum, were used for rooting experiments. Roots were produced in 50–70% of the regenerated peach shoots on half-strength MS inorganic medium containing 2.5–5.0 μM IBA in light (16 h photoperiod) within 14–28 days. The roots were stubby (> 1 mm thick) and varied in number from one to six (data not presented).

Regenerated shoots of plum were cultured on half-strength MS inorganic medium without growth hormones either in light (16 h photoperiod) or in darkness at 24 °C for 3 weeks and then subcultured onto the same medium with 2.5–5.0 μM IBA in light. The dark-treated shoots were slightly etiolated prior to culturing in the presence of IBA. In both treatments, 20–25% of the regenerated plum shoots rooted.

Acclimatization of rooted plants

Rooted peach and plum plants were transferred into Plant Cons (Flow Labs., McLean, VA, USA), containing a 1:1 peat moss:perlite mix. They were fertilized weekly with half-strength MS inorganic solution for 2 weeks before transfer to the greenhouse. The survival rate was 90% (Fig. 3). Regenerated plants (9 cultivars) established in soil were stable, and there was no apparent phenotypic difference between them and greenhouse grown *Prunus* seedlings.

Fig. 3. 120-day-old regenerated plants, plum (left) and peach (right) growing in 15.2 cm pots. Bar = 5 cm.

Discussion

The cytokinin-like activity of TDZ has been documented in a number of plant tissue culture systems [3, 7, 14]. Our report indicates that this compound is effective for shoot organogenesis from cotyledons of various peach cultivars, European plum, and sour cherry. Shoot regeneration from cotyledons was entirely dependent upon the removal of the embryonic axis and the presence of the proximal region of the cotyledon. The removal of the embryonic axis from its point of attachment probably produced a cut

surface on the cotyledons; it is not known if this influenced shoot morphogenesis. Shoot development did not occur if the proximal region was excised. A similar observation was made in apple cotyledons [12]. In the absence of the embryonic axis, the area of attachment on the cotyledon appeared to be stimulated to develop into either shoots or roots, depending upon the presence of exogenous growth regulators.

In plum and sour cherry the shoot morphogenic capacity was evident in mature cotyledons, as had also been demonstrated in apple [12]. We have observed the same phenomenon in *Pyrus communis* (unpubl.). However, in peach, the frequency of shoot formation decreased with increasing age of the cotyledon. A similar observation was made with peach embryogenic calli derived from immature embryos [4]. It is not known whether the storage of immature peach fruit at 4 °C, up to 90 days, influenced adventitious shoot development, but it significantly extended the length of time that explants were available for investigations.

The ability to reliably regenerate plants from *Prunus* cotyledons has a number of practical applications. The method takes about 100 days from initial culture to the establishment of rooted plants in the greenhouse. Shoot regeneration from cotyledons is faster and requires less steps than regeneration from embryo-derived callus [4]. The culture of immature peach cotyledons may provide a more efficient means of producing plants of early ripening cultivars in vitro. These plants do not develop mature embryos in vivo and require a multi-step process of in vitro culture and stratification to produce each seedling [11]. In contrast, adventitious shoot regeneration from cotyledons requires less manipulation in vitro and multiple shoots are produced per seed. Other potential applications of this methodology include the culture of immature cotyledons of interspecific *Prunus* hybrids, and the improvement of *Prunus* rootstocks through the introduction of engineered genes.

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